0022-202X/78/7102-0154$02.00/0
THE JOURNAL OF INVESTIGATIVE DERMATOLOGY, 71:154–156, 1978

Vol. 71, No. 2
Printed in U.S.A.

Papaverine and Ro 20-1724 Inhibit Cyclic Nucleotide Phosphodiesterase Activity and Increase Cyclic AMP Levels in Psoriatic Epidermis In Vitro

LOUIS J. RUSIN, M.D., ELIZABETH A. DUELL, Ph.D., AND JOHN J. VOORHEES, M.D.

Department of Dermatology, University of Michigan Medical School, Ann Arbor, Michigan, U.S.A.

The comparative inhibitory potency of papaverine and Ro 20-1724 (4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone) on cyclic AMP-phosphodiesterase (cAMP-PDE) and cyclic GMP-phosphodiesterase (cGMP-PDE) activities and their effect on the levels of cAMP and cGMP were examined in psoriatic epidermis. At concentrations of 5×10^{-4} M, papaverine inhibited the hydrolysis of both cAMP and cGMP by either the low or high Km psoriatic epidermal PDE nearly 100% ($p < .0001$) while Ro 20-1724 selectively inhibited the hydrolysis of cAMP 94% ($p < .0001$) but had no significant effect on cGMP hydrolysis. When keratomed psoriatic epidermal slices were incubated in 5×10^{-4} M papaverine or Ro 20-1724 the tissue levels of cAMP were increased 343% or 1395% respectively ($p < .001$) with no concomitant change in the levels of cGMP. Selectice inhibition of cAMP hydrolysis by Ro 20-1724 and its greater effectiveness in elevating cAMP levels in slices of psoriatic epidermis is one explanation for its clinical superiority in treating psoriatic lesions.

Lesions of psoriatic epidermis are characterized by accelerated cell turnover [1], incomplete cellular differentiation [2], and glycogen accumulation [3]. These abnormal characteristics may be in part the result of misregulated lesional cyclic nucleotide metabolism [4–7].

It is known that in nonepidermal tissues papaverine inhibits cyclic AMP-phosphodiesterase (cAMP-PDE) and cGMP-PDE whereas 4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone (Ro 20-1724) only inhibits cAMP-PDE [8–14]. Both agents improve psoriatic lesions; Ro 20-1724 significantly better than papaverine [15,16]. This paper presents evidence that papaverine and Ro 20-1724 differentially inhibit psoriatic epidermal cyclic nucleotide phosphodiesterases and elevate cyclic nucleotide levels in involved and uninvolved psoriatic epidermis in vitro.

MATERIALS AND METHODS

Materials

3′,5′-cyclic adenosine monophosphate (cAMP), 3′,5′-cyclic guanosine monophosphate (cGMP), and 5′nucleotidase were purchased from Sigma Chemical Company. ^{3}HcAMP and ^{3}HcGMP were purchased from New England Nuclear. Papaverine hydrochloride was a gift from Lilly Corporation and Ro 20-1724 from Hoffman-LaRoche. Antibodies to cAMP and cGMP were a gift from Dr. Cynthia Marcelo, Department of Dermatology, University of Michigan. The iodinated tyrosine methyl esters of cAMP and cGMP were obtained from Collaborative Research. All other chemicals were reagent grade and obtained from common commercial sources. Distilled, deionized water was used for the preparation of all solutions.

Methods

The tissue for the isolation of the cyclic nucleotide PDE activities and for the incubations with the 2 inhibitors was obtained from psoriasis patients with a Castroviejo electrokeratome. The depths of the biopsies were 0.375 or .400 mm in involved and 0.125 mm for uninvolved areas. Epidermal purity was monitored histologically as previously described [4]. To measure the activity of cyclic nucleotide PDE, epidermal specimens were immediately placed in liquid nitrogen, weighed, pulverized in a mortar and pestle under liquid nitrogen, and homogenized (300 mg epidermal powder/ml) in ice-cold Tris-HCl buffer (0.04 M, pH 7.5) in a motor driven Ten Broeck homogenizer. Homogenates were centrifuged at 17,000 ×g for 30 min and the supernatant divided into aliquots which were stored at −70°C. Soluble protein in the supernatant was quantitated by the method of Lowry et al [17].

PDE activity was measured by the method of Boudreau and Drummond [18]. The standard incubation mixture contained 10 mM Tris-HCl, pH 7.5, 1 mM $MgCl_2$, ^{3}H-cAMP or ^{3}H-cGMP, cAMP or cGMP, Ro 20-1724 or papaverine as indicated, and H_2O to a final volume of 200 μl. Aliquots of the 17,000 ×g supernatant were added to initiate the reaction. Following 5, 10, 15, and 20 min incubation at 30°C, 50 μl aliquots were removed and the reaction terminated by boiling for 5 min. Following reequilibration to 30°C, 10 μl of a 10 mg/ml solution of 5′-nucleotidase was added to each tube. After a 20-min incubation the reaction was terminated by the addition of 1.0 ml of 25% resin slurry (AG 1-X2, 200-400 mesh). The resin slurry for cAMP-PDE activity contained 3.0 mM acetic acid to give a pH of 3.00 while the resin for cGMP contained formic acid, pH 2.25. The tubes were allowed to stand at least 10 min then centrifuged at 100 ×g for 10 min. Aliquots of 0.5 ml were placed in scintillation vials and counted in a dioxane based scintillation fluid. The degree of PDE inhibition was determined by comparing the amount of ^{3}H-adenosine or ^{3}H-guanosine formed in the presence of Ro 20-1724 or papaverine to the control values in the absence of these drugs.

More than 95% of ^{3}H-adenosine and 90% of ^{3}H-guanosine could be recovered by this method. Less than 5% of the tritiated nucleotides remained in solution when the appropriate acetic acid or formic acid resin slurry was added to the initial reaction mixture. Less than 10% of the cyclic nucleotide added as substrate was hydrolyzed during the incubation. All assays were linear as a function of time and protein concentration.

For the *in vitro* incubation with inhibitors, the tissues were minced into 3-mm squares and pre-incubated for 15 min at 37° in Krebs-Ringer bicarbonate, pH 7.5, with 0.5% glucose. At zero time papaverine or Ro 20-1724 were added to experimental flasks to give a 5×10^{-4} M final concentration of inhibitor. After 15 min of incubation the tissue was removed and immediately placed in liquid nitrogen. Fifteen min is the optimal time to observe the effect of PDE inhibition by papaverine or Ro 20-1724 (unpublished results obtained with hairless mouse epidermis, L.J. Rusin). The slices were subsequently pulverized in a mortar and pestle under liquid nitrogen and then homogenized in 5 ml of 6% trichloracetic acid containing tracer ^{3}H-cAMP and ^{3}H-cGMP for calculation of cyclic nucleotide recovery. Partial purification of the cyclic nucleotides was performed by co-precipitation of a majority of the noncyclic nucleotides with 1.0 M zinc acetate-2.0 M sodium carbonate, followed by Dowex 50 × 8 (100-200 mesh, H^+) and Dowex 1 × 2 (200-400 mesh, Cl^-) ion exchange chromatography. The fractions containing cAMP and cGMP were lyophilized and acetylated prior to radioimmunoassay by the method of Harper and Brooker [19]. The cyclic nucleotide content was measured by a minor modification of the

Manuscript received for publication November 18, 1977; accepted for publication February 15, 1978.

Presented at the Residents' Forum 35th, Annual Meeting of The American Academy of Dermatology, Chicago, Ill., December 6, 1976.

This investigation was supported by grant 5 P01-AM 15740 from the NIAMDD branch of the NIH.

Reprint requests to: Louis J. Rusin, M.D., Department of Dermatology, University of Michigan Medical School, 5548 Kresge Research Building I, Ann Arbor, MI 48109.

Abbreviations:
cAMP: 3′,5′-cyclic adenosine monophosphate
cGMP: 3′,5′-cyclic guanosine monophosphate
cAMP-PDE: cAMP-phosphodiesterase
cGMP-PDE: cGMP-phosphodiesterase
5′AMP: 5′ adenosine monophosphate
5′GMP: 5′ guanosine monophosphate
Ro 20-1724: 4(3-butoxy-4-methoxybenzyl)-2-imidazolidinone

radioimmunoassay of Steiner, Parker, and Kipnis [20]. Quality control of these cyclic nucleotide radioimmunoassays was as follows: (1) duplicate assays of triple dilutions were ± 10%; (2) removal of reactive material by PDE digestion prior to acetylation; (3) internal standards performed at the beginning and end of each radioimmunoassay.

Data was analyzed using the Kruskal-Wallis nonparametric test for multisample comparisons.

RESULTS

Inhibition of Soluble Epidermal Cyclic Nucleotide PDE by Papaverine or Ro 20-1724

The hydrolysis of cyclic AMP or cyclic GMP was linear with time (5–45 min of incubation). The data obtained at the 15-min time point are summarized in Fig 1. Papaverine inhibited the hydrolysis of cyclic AMP or cyclic GMP nearly 100% ($p < 0.0001$) when either the high or low concentrations of cyclic AMP or cyclic GMP were present in the reaction mixture. In contrast, Ro 20-1724 inhibited cyclic AMP hydrolysis 94% ($p < 0.0001$) only at low substrate concentrations and was ineffective at high substrate concentrations. No significant inhibition of cyclic GMP hydrolysis could be demonstrated at either high or low substrate concentrations.

The inhibitory dose 50 (ID_{50}) for papaverine and Ro 20-1724 with 2×10^{-6} M cyclic AMP as substrate is 2.0×10^{-5} M and 3.5×10^{-5} M, respectively. The ID_{50} for papaverine with 5×10^{-6} M cyclic GMP as substrate is 3.5×10^{-5} M.

Accumulation of Cyclic Nucleotides in Sliced Preparations Incubated with Papaverine or Ro 20-1724

The results of the radioimmunoassay of cyclic nucleotides in psoriatic epidermal slices, expressed as picomoles of cyclic AMP per μg of tissue DNA [21] are given in Fig 2. The increase in cyclic AMP in the uninvolved psoriatic epidermis was 389% in the presence of papaverine while Ro 20-1724 increased the levels by 1200%. In the involved epidermis there was a 387% increase in cyclic AMP levels in the presence of papaverine and 711% when Ro 20-1724 was the additive ($p < 0.001$, $N = 8$). Similar results were obtained if the protein content of the tissue served as the data base (388% increase with papaverine, 1131% increase with Ro 20-1724 in the uninvolved epidermis; 343% increases with papaverine, 1395% with Ro 20-1724 in the involved tissue, $p < 0.001$, $N = 8$). The increases in cyclic AMP content were also similar with wet weight as a data base. A statistically significant ($p < 0.005$) increase in cyclic AMP levels was observed when the cyclic AMP levels obtained with the addition of Ro 20-1724 were compared to levels obtained with the addition of papaverine regardless of the data base. Neither Ro 20-1724 nor papaverine significantly altered the tissue levels of cyclic GMP in the incubated slices.

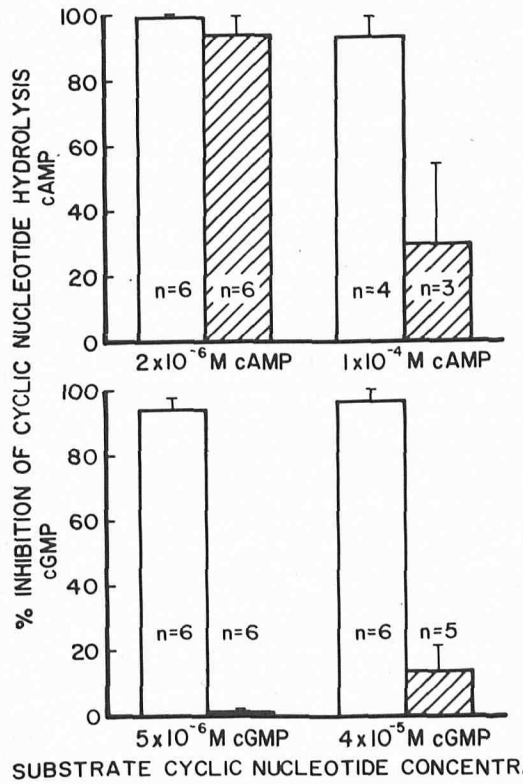

FIG 1. Inhibition of cyclic nucleotide hydrolysis with 5×10^{-4} M papaverine (*open bars*) or 5×10^{-4} M Ro 20-1724 (*hashed bars*). The control rates are represented as 0% in the figure. With low or high substrate concentrations papaverine significantly inhibited cAMP hydrolysis 99.8% or 94.8% respectively ($p < .0001$). Ro 20-1724 significantly inhibited cAMP hydrolysis (94.2%, $p < .0001$) only with a low substrate cAMP concentration and did not significantly inhibit cGMP hydrolysis. Results are means of epidermal samples obtained from 3–6 patients with psoriasis.

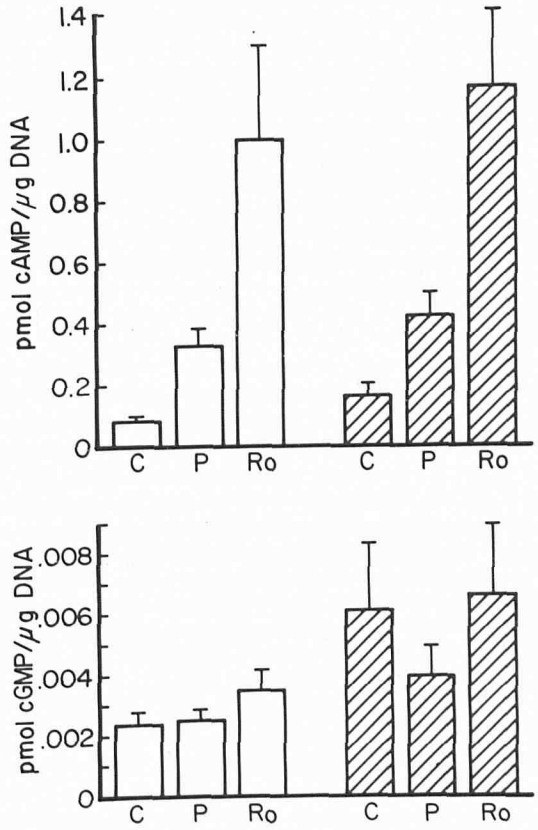

FIG 2. Cyclic AMP or cyclic GMP accumulation in psoriatic epidermis. Epidermal slices were incubated as described in the text, with 5×10^{-4} M papaverine (*P*) or Ro 20-1724 (*Ro*) or with buffer alone (*c*). The *open bars* represent uninvolved psoriatic epidermis; the *hashed bars* involved psoriatic epidermis. Results are means of epidermal samples obtained from 8 patients with psoriasis and *bars* indicate the SEM. Papaverine or Ro 20-1724 significantly increased cAMP accumulation in involved or uninvolved tissue ($p < .001$) but had no significant effect on cGMP accumulation.

DISCUSSION

As in other tissues [9,22–23] cyclic nucleotide PDE activity in the skin of rat [24], mouse [25–26], pig [27], and human [25] is both soluble and particulate. Furthermore, 65–75% of the cAMP-PDE [24–27] and all of the detectable cGMP-PDE [27] activities are found in the soluble epidermal fraction. Kinetic studies of epidermal cAMP-PDE and cGMP-PDE indicate that both enzymes have 2 apparent Km values [24,25,27,28]. We have shown that Ro 20-1724 selectively inhibits hydrolysis of cAMP by the low Km cAMP-PDE in the soluble fraction from

involved psoriatic epidermis. Papaverine inhibits both the high and low Km cAMP-PDE and cGMP-PDE. These data suggest that Ro 20-1724 may have the potential to selectively increase cAMP in psoriatic lesions (by decreasing its breakdown) whereas papaverine may increase both cAMP and cGMP levels resulting in an unpredictable and possibly undesirable change in the ratio of tissue cAMP/cGMP.

Papaverine or Ro 20-1724 significantly increased the accumulation of cAMP in incubated slices of involved or uninvolved psoriatic epidermis. Ro 20-1724 increased cAMP accumulation to a significantly larger extent than papaverine. Neither papaverine nor Ro 20-1724 increased the accumulation of cGMP. The results obtained with the soluble enzyme suggest that Ro 20-1724 would selectively elevate the tissue levels of cAMP while papaverine would elevate the levels of both cAMP and cGMP. Papaverine did not elevate cGMP levels in the sliced preparations. If low levels of cGMP are present in the incubated tissue then this could be rate-limiting for the cGMP-PDE and a PDE inhibitor would be ineffective in elevating the tissue levels of cGMP. The hypothesis that cGMP is rate-limiting is supported by the observations in incubated hairless mouse epidermis where the levels of cAMP are 20 times higher than cGMP (.005–.007 picomoles cGMP/μg tissue DNA compared to .10–.15 picomoles cAMP/μg DNA*). Much larger concentrations of cGMP (5×10^{-6} M—4×10^{-5} M) were used in the broken-cell assay of PDE-activity and therefore an inhibition of cGMP-PDE by papaverine could be observed.

Both compounds are equally effective in inhibiting *in vitro* cAMP-PDE activity and we have no good explanation for the superiority of Ro 20-1724 in elevating the cAMP levels in psoriatic slices. Perhaps the lipophilic character of Ro 20-1724 [29] contrasted with the more marked water solubility of papaverine, may permit greater penetration of Ro 20-1724 into cells. Alternatively, Ro 20-1724 may inhibit the tissue equivalent of the particulate form of cAMP-PDE (25–35% of the total activity) to a greater extent than papaverine or stimulate cell membrane-bound adenylate cyclase which was removed in the broken-cell assay of PDE-activity.

Selective inhibition of cAMP hydrolysis by Ro 20-1724 and its greater effectiveness in elevating cAMP levels in slices of psoriatic epidermis is one explanation for its superiority in the clinical psoriasis bioassays [16]. These data are compatible with the hypotheses that enhancing the net effects of cAMP in psoriatic epidermis may improve the lesion. Examining the effects of agents on cyclic nucleotide PDE-activity and ability to alter tissue levels of cAMP and cGMP may be useful in selecting other agents with therapeutic potential in the management of psoriasis and other proliferative skin diseases.

The expert technical assistance of Victoria Wozny in the cyclic nucleotide determinations is greatly appreciated.

* Unpublished results by Cynthia Marcelo.

REFERENCES

1. Weinstein GD, McCullough JL: Cytokinetics in diseases of epidermal hyperplasia, Annual Review of Medicine, vol 24. Edited by WP Creger. Palo Alto, Annual Reviews, 1973, pp 345–352
2. Van Scott EJ: Tissue compartments of the skin lesion of psoriasis. J Invest Dermatol 59:4–6, 1972
3. Halprin KM, Ohkawara A: Carbohydrate metabolism in psoriasis: An enzymatic study. J Invest Dermatol 46:51–68, 1966
4. Voorhees JJ, Duell EA, Bass LJ, Powell JA, Harrell ER: Decreased cyclic AMP in the epidermis of lesions of psoriasis. Arch Dermatol 105:695–701, 1972
5. Voorhees JJ, Stawiski M, Duell EA, Haddox MK, Goldberg ND: Increased cyclic GMP and decreased cyclic AMP levels in the hyperplastic abnormally differentiated epidermis of psoriasis. Life Sci 13:639–653, 1973
6. Harkonen M, Hopsu-Hauer VK, Raij K: Cyclic adenosine monophosphate, adenyl cyclase and cyclic nucleotide phosphodiesterase in psoriatic epidermis. Acta Derm Venereol (Stockh) 54:13–18, 1974
7. Yoshikawa K, Adachi K, Levine V, Halprin KM: Micro-determination of cyclic AMP levels in human epidermis, dermis, and hair follicles. Br J Dermatol 92:241–248, 1975
8. Lugnier C, Stoclet J: Inhibition of papaverine of cGMP and cAMP phosphodiesterases from the rat heart. Biochem Pharmacol 23:3071–3074, 1974
9. Fertel R, Weiss B: Properties and drug responsiveness of cyclic nucleotide phosphodiesterase of rat lung. Mol Pharmacol. 12:678–687, 1976.
10. Wells, YN, Wu YJ, Baird CE, and Hardman JG: Phosphodiesterases from porcine coronary arteries: Inhibition of separated forms by xanthines, papaverine, and cyclic nucleotides. Mol Pharmacol 11:775–783, 1975
11. Adachi K, Numano F: Phosphodiesterase inhibitors: Their comparative effectiveness in vitro in various organs. Jap J Pharmacol 27:97–103, 1977
12. Goff AK, Major PW: Bovine luteal cyclic AMP and cyclic GMP phosphodiesterase activity; Sensitivity to various drugs and hormones. FEBS Lett 61:144–147, 1976
13. Bergstrand H, Kristofferson J, Lundquist B, Schurmann A: Effects of an antiallergic agents, compound 48/80, and some reference inhibitors on the activity of partially purified human lung tissue adenosine cyclic 3′,5′-monophosphate and guanosine 3′,5′-monophosphate phosphodiesterases. Mol Pharmacol 13:38–43, 1977
14. Prasad KN, Becker G, Tripathy K: Differences and similarities between Guanosine 3′,5′-cyclic monophosphate phosphodiesterase and adenosine 3′,5′-cyclic monophosphate phosphodiesterase activities in neuroblastoma cells in culture (38893) PSEBM 149:757–762, 1975
15. Stawiski MA, Powell JA, Lang PG, Schork A, Duell EA and Voorhees JJ: Papaverine: Its effects on cyclic AMP *in vitro* and psoriasis *in vivo*, J Invest Dermatol 64:124–127, 1975
16. Stawiski MA, Rusin LJ, Schork MA, Burns T, Duell EA, Voorhees JJ: Ro 20-1724 elevates epidermal cAMP levels *in vitro* and improves psoriasis *in vivo*. Clin Res 24:267, 1976
17. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ: Protein measurement with the folin phenol reagent. J Biol Chem 193:265–275, 1951
18. Boudreau RJ, Drummond GI: A modified assay of 3′:5′-cyclic-AMP phosphodiesterase. Anal Biochem 63:388–399, 1975
19. Harper JF, Brooker G: Femtomole sensitive radioimmunoassay for cyclic AMP and cyclic GMP after 2′0 acetylation by acetic annhydride in aqueous solution. J Cyclic Nuc Res 1:207–218, 1975
20. Steiner AL, Parker CW, Kipnis DM: Radioimmunoassay for cyclic nucleotides. J Biol Chem 247:1106–1113, 1972
21. Burton K: Determination of DNA concentration with diphenylamine, Methods in Enzymology, vol 12, part B. Edited by L Grossman, K Moldave. New York, Academic Press, 1968, pp 163–165
22. Beavo JA, Hardman JG, Sutherland EW: Hydrolysis of cyclic guanosine and adenosine 3′,5′-monophosphates by rat and bovine tissues. J Biol Chem 245:5649–5655, 1970
23. DeRoberts E, Rodriquez de Lores Arnaiz G, Alberici M, Butcher RW, Sutherland EW: Subcellular distribution of adenyl cyclase and cyclic phosphodiesterase in rat brain cortex. J Biol Chem 242:3487–3493, 1967
24. King LE, Solomon SS, Hashimoto K: Cyclic 3′,5′-nucleotide phosphodiesterase in rat skin. J Invest Dermatol 64:390–396, 1975
25. Marks F, Raab I: The second messenger system of mouse epidermis. Biochim Biophys Acta 334:368–377, 1974
26. Mier PD, Urselmann E: Adenosine 3′,5′-cyclic monophosphate phosphodiesterase in skin. Br J Dermatol 86:141–147, 1971
27. Adachi K, Levine V, Halprin KM, Iizuka H, Yoshikawa K: Multiple forms of cyclic nucleotide phosphodiesterase in pig epidermis. Biochim Biophys Acta 429:498–507, 1976
28. Birnbaun JE, Sapp TM, Jolman EL: Cyclic AMP-phosphodiesterase and epidermal mitosis. J Invest Dermatol 67:235–239, 1976
29. Shultz J: Inhibition of 3′,5′-nucleotide phosphodiesterase in guinea pig cerebral cortical slices. Arch Biochem Biophys 163:15–20, 1974